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Modulating Cell-Surface Receptor Signaling and Ion Channel Functions by In Situ Glycan Editing

Dr. Hao Jiang,

Key Laboratory of Marine Drugs, Ministry of Education and Qingdao National Laboratory for Marine Science & Technology and Shandong Provincial Key Lab of Glycoscience & Glycoengineering, School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao, 266003 (China)

Dr. Aimé López-Aguilar,

Department of Molecular Medicine, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037 (USA)

Dr. Lu Meng,

Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, 315 Riverbend Road, Athens, GA 30602 (USA)

Dr. Zhongwei Gao,

Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, 315 Riverbend Road, Athens, GA 30602 (USA)

Dr. Yani Liu,

Department of Pharmacology, School of Pharmacy, Qingdao University, 38 Dengzhou Road, Qingdao, 266021 (China)

Xiao Tian,

Key Laboratory of Marine Drugs, Ministry of Education and Qingdao National Laboratory for Marine Science & Technology and Shandong Provincial Key Lab of Glycoscience & Glycoengineering, School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao, 266003 (China)

Prof. Guangli Yu,

Key Laboratory of Marine Drugs, Ministry of Education and Qingdao National Laboratory for Marine Science & Technology and Shandong Provincial Key Lab of Glycoscience & Glycoengineering, School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao, 266003 (China)

Dr. Ben Ovryn,

Department of Molecular Medicine, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037 (USA)

Correspondence to: Hao Jiang; Peng Wu.

Conflict of interest

The authors declare no conflict of interest.

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Prof. Kelley W. Moremen, and

Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, 315 Riverbend Road, Athens, GA 30602 (USA)

Prof. Peng Wu

Department of Molecular Medicine, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037 (USA)

Abstract

Glycans anchored on cell-surface receptors are active modulators of receptor signaling. A strategy is presented that enforces transient changes to cell-surface glycosylation patterns to tune receptor signaling. This approach, termed *in situ* glycan editing, exploits recombinant glycosyltransferases to incorporate monosaccharides with linkage specificity onto receptors *in situ*. α 2,3-linked sialic acid or α 1,3-linked fucose added *in situ* suppresses signaling through epidermal growth factor receptor and fibroblast growth factor receptor. We also applied the same strategy to regulate the electrical signaling of a potassium ion channel–human ether-à-go-go-related gene channel. Compared to gene editing, no long-term perturbations are introduced to the treated cells. *In situ* glycan editing therefore offers a promising approach for studying the dynamic role of specific glycans in membrane receptor signaling and ion channel functions.

Fucose a go-go

Monosaccharides added *in situ* actively tune cell-surface receptor signaling and ion channel properties. α 2,3-linked sialic acid or α 1,3-linked fucose suppress signaling through the epidermal and fibroblast growth factor receptor. *In situ* glycan editing offers a promising approach for studying the dynamic role of specific glycans in membrane receptor signaling and ion channel functions.

Keywords

click chemistry; fucosylation; *in situ* glycan editing; sialylation

Upon ligand binding, receptors embedded in the plasma membrane transduce messages into intracellular signaling molecules in a cascade of events, leading to changes in cell behavior. By combining the specificity of genetic manipulation and the spatiotemporal resolution of light- or small molecule-based approaches, it is possible to control receptor signaling in cultured cells or in living organisms. Elegant examples include photoswitchable ion channels controlled by azobenzene^[1] and “on-switch” chimeric antigen receptor T cells tuned by a rapamycin analogue.^[2]

Essentially all membrane receptors are glycosylated and the anchored glycans actively mediate receptor signaling by modulating ligand–receptor binding, receptor dimerization, endocytosis, and degradation.^[3] In many instances the role of specific glycans in these processes remains obscure despite the new advances in gene editing^[4] and metabolic oligosaccharide engineering (MOE).^[5] MOE remodels glycan structures by supplementing the growth media with carbohydrate analogues that get incorporated into glycans by the endogenous biosynthetic machinery.^[6] To our knowledge there has been only one report from our previous study where MOE was employed to control a specific signaling pathway, in which we discovered that enhanced fucosylation on N-glycans inhibits Wnt signaling.^[7]

Nevertheless, modification of cell-surface glycans using genetic or MOE approaches are not free of limitations: due to the functional redundancy of many glycosyltransferases, typically more than one gene needs to be knocked out in order to produce a phenotype,^[8] and gene editing of glycosyltransferases may also introduce unexpected side effects, for example, chaperone functions.^[9] Likewise, MOE often leads to changes on diverse array of glycans. Therefore, there is a critical need to explore alternative approaches for the modification of cell-surface glycans.

Herein, we report a method, termed in situ glycan editing, to control receptor signaling and ion channel functions and demonstrate that it serves as a straightforward and fast approach to modify glycocalyx (Figure 1). In situ glycan editing finds its roots in chemoenzymatic glycan labeling which has been exploited by several laboratories, including our own, to visualize cell-surface higher order glycans. In chemoenzymatic glycan labeling, which has been successfully used to visualize N-acetyllactosamine (LacNAc), fucose α 1,2-galactose, TF antigen, among a few others,^[10] a recombinant glycosyltransferase is used to transfer a monosaccharide bearing a chemical tag from the corresponding nucleotide sugar donor to a cell-surface acceptor glycans in situ. The tag can be derivatized using bioorthogonal click chemistry to install a biophysical probe for imaging.

In this study (Figure 1), we demonstrate that in situ glycan editing using human α 2,3-sialyltransferase ST3Gal-IV when combined with bioorthogonal click chemistry, provides a facile method to validate the identity of membrane receptors modified by the in situ added monosaccharides. We discover that the newly added sialic acid or fucose (using *H. pylori* α 1,3-fucosyltransferase– α 1,3 FucT)^[11] without further derivatization actively tune the strength of membrane receptor signaling and ion channel functions.

ST3Gal-IV, one of the 20 Golgi-resident sialyltransferases annotated in humans, controls sialyl Lewis X biosynthesis on N- and O-glycans in cells of myeloid lineage.^[12] In vivo

ST3Gal-IV is known to add sialic acids onto the terminal galactose of N- and O-glycans,^[13] however, the in vitro specificity and substrate scope of recombinant human ST3Gal-IV for cell-surface glycan editing has not been characterized. Kinetic analysis using type II LacNAc as the acceptor substrate revealed that this enzyme has excellent activity toward the natural donor substrate CMP-Neu5Ac (CMP-Sia) with K_m of 0.0734 mM and V_{max} of 0.0478 nmol min⁻¹. The enzyme can also accept an alkyne modified CMP-Sia analogue (CMP-SiaNAI) as the donor with a slightly lower activity (K_m of 0.211 mM and V_{max} of 0.0404 nmolmin⁻¹; Supporting Information, Figure S1a,b).

To assess the ability of the recombinant ST3Gal-IV to modify glycocalyx, we treated Lec2 Chinese hamster ovary (CHO) cell mutants that express abundant peripheral LacNAc disaccharides^[14] with CMP-SiaNAI and ST3Gal-IV. At various time points we subjected the ST3Gal-IV treated cells to the ligand accelerated copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC)^[15] to install biotin tags to the newly added SiaNAI (Figure 1). The biotinylated cells were then probed with Alexa Fluor 488-streptavidin enabling flow cytometry and confocal microscopy analysis. In situ Sia editing produced robust fluorescent signals in Lec2 cells whereas control Lec8 cells lacking terminal galactose only showed background labeling (Supporting Information, Figure S1c), and the endogenous sialyltransferase had no contribution to in situ Sia editing on Lec2 cell surface (Supporting Information, Figure S2a). After a 40 min in situ Sia editing reaction (600 μ M of CMP-SiaNAI and 50 μ g mL⁻¹ of ST3Gal-IV), the cell-associated fluorescence reached a plateau, suggesting the saturation of cell-surface accessible sialylation sites (Supporting Information, Figure S1c–e). The half-life of the newly added sialic acid on the cell surface was approximately two hours (Supporting Information, Figure S1f).

To determine ex vivo cell-surface behavior of the recombinant enzyme, we performed in situ Sia editing using three CHO cell lines, Lec2, Lec1, and Lec8; these mutants are known to have distinct glycosylation patterns: Lec2 expresses galactose-terminated N-glycans and the unsialylated core 1 O-glycan Gal- β -(1-3)-GalNAc; Lec1 does not synthesize complex or hybrid N-glycans, but its O-glycans synthesis is not affected; and Lec8 expresses complex type N-glycans and core 1 O-glycans lacking the terminal galactose.^[14] In situ Sia editing was performed by treating all three cell lines with CMP-SiaNAI and ST3Gal-IV, then newly added SiaNAI was labeled by biotin via CuAAC. The labeled cells were either lysed for western blot analysis or stained with Alexa Fluor 488-streptavidin for flow cytometry or confocal microscopic imaging. Lysates from Lec2 cells treated with CMP-SiaNAI exhibited robust signal that was abolished upon PNGase-F mediated N-glycan removal. By contrast, signals were not detected in lysates from Lec1 and Lec8 cells (Figure 2a). This result was consistent with flow cytometry analysis (Figure 2b) and confocal imaging (Figure 2c). Together, these observations suggest that ex vivo ST3Gal-IV preferentially modifies N-linked glycans, leaving O-glycans unperturbed in CHO cells.

After identifying the substrate scope and specificity of in situ Sia editing, we next sought to apply this method to functional study of cell surface proteins. We selected three model systems that have been shown to have their function influenced by their glycosylation state. Epidermal growth factor receptor (EGFR) possesses eleven N-linked glycosylation sites with potential sialylation or fucosylation,^[16] and performs critical roles in essential cellular

processes which control cell proliferation and migration. Mutations in the EGFR gene are associated with pathogenesis and progression of different carcinoma types, in particular those of pulmonary origin.^[17] Wong et al. found that overexpression of α 2,3-sialyltransferases and α 1,3-fucosyltransferases in lung cancer cells suppress EGF-induced receptor dimerization and phosphorylation.^[3b] However, enzyme overexpression usually causes permanent perturbation to the treated cells. To overcome this drawback we sought to determine if adding sialic acid or fucose directly on the cell surface via the in situ glycan editing strategy may convey similar effects. Through this approach only transient perturbation is introduced (Supporting Information, Figure S1 f); cell viability assay indicated that the in situ glycan editing is non-toxic to the treated cells (Supporting Information, Figure S3). Toward this end, we incubated the adenocarcinoma human alveolar basal epithelial cell A549, a cell line expressing high levels of EGFR, with ST3Gal-IV and CMP-SiaNAI to determine if cell-surface EGFR could be modified. Treated cell lysates were biotinylated and immunoprecipitated using anti-EGFR or anti-biotin. Western blot analysis indicated that immunoprecipitated EGFR was modified by biotin and the enriched biotinylated protein pools contained EGFR, while the control sample which was subjected to ST3Gal-IV in the presence of CMP-Sia showed no detectable signal (Figure 3a). These results confirmed that EGFR were indeed modified by in situ Sia editing.

Next, we evaluated if in situ Sia or Fuc editing could alter EGFR dimerization, activation or degradation. A549 cells were subjected to in situ Sia editing using CMP-Sia as the glycan donor or in situ Fuc editing using GDP-fucose (GDP-Fuc) as the glycan donor, followed by EGF stimulation. Western blotting analysis of the cell lysates indicated that the formation of the EGFR dimer decreased upon in situ Sia or Fuc editing by 37% and 25%, respectively (Figure 3b). Similarly, Tyr 1068 phosphorylation of EGFR was reduced by 36% and 63% in each case (Figure 3b). In the control experiment where cells were only treated with ST3Gal-IV without the CMP-Sia donor, no suppression of EGFR dimerization was observed (data not shown). Interestingly, using CMP-SiaNAI as the in situ donor resulted in more pronounced inhibition of EGFR dimerization (Supporting Information, Figure S4). It is known that the EGF-stimulated mitogen-activated protein kinase (MAPK) signaling occurs primarily in the plasma membrane and that signaling through EGFR induces the activation of MAPK by phosphorylation at Tyr1068.^[18] Consistent with the suppressive role of in situ Sia or Fuc editing on EGFR activation, we also observed the decreased phosphorylation of MAPK by 35% and 29%, respectively (Figure 3b). To confirm that these observations were indeed caused by in situ glycan editing of EGFR, the above experiments were repeated via in situ Fuc editing in the presence of the EGFR inhibitor gefitinib. An additive effect was detected, verifying EGFR-suppression was indeed caused by in situ Fuc editing (Supporting Information, Figure S5). Surprisingly, despite the pronounced impact of in situ Sia editing on EGF-induced EGFR dimerization and activation, little influence of this treatment on EGF-induced EGFR degradation was observed (Supporting Information, Figure S6).

Previous studies showed that signaling through EGFR enhances the migration of cancer cells.^[19] To test if in situ Sia editing has any impact on EGFR dependent migration, a wound healing assay was performed on the EGF treated A549 cells. As shown in Figure 3c, in situ Sia editing of EGF stimulated cancer cells showed significantly suppressed cell migration, but did not impact non-stimulated cancer cells. To further validate this result, we treated the

cells with a known metabolic inhibitor of sialyltransferases, 2,4,7,8,9-pentaacetyl-3Fax-Neu5Ac-CO₂Me to block the biosynthesis of sialylated glycans,^[20] and measured the EGF-mediated cell migration. As a result, a significant increase of cell migration was observed in the inhibitor treated cells as compared with untreated or in situ Sia edited cells (Figure 3c). Similar effects were also observed in in situ Fuc edited and defucosylated A549 cells, and the impact of fucosidase-mediated defucosylation could be rescued by in situ Fuc editing (Supporting Information, Figure S7).

To evaluate if in situ glycan editing can serve as a general approach to control receptor signaling, we examined its impact on fibroblast growth factor receptor (FGFR)-mediated signaling. FGF-FGFR complex comprises two receptor molecules, two FGFs and a co-receptor heparin.^[21] Aberrant FGF signaling can promote tumor development by directly driving cancer cell proliferation and supporting tumor angiogenesis.^[22] It is reported that FGFR signaling is regulated by its O- and N-glycosylation.^[23] Here we chose the human breast adenocarcinoma cell line MCF-7 that expresses FGFR to evaluate the efficacy of our strategy. MCF-7 cells were subjected to in situ Sia editing followed by stimulation with FGF and heparin. FGFR dimer formation and the downstream phosphorylation of MAPK were significantly suppressed upon in situ Sia editing (by 26% and 58%, respectively; Supporting Information, Figure S8a,b) compared to the control unsialylated cells. Similarly, FGFR-mediated activation of MAPK was also impaired by 47% upon in situ Fuc editing (Supporting Information, Figure S8b).

As the initial evaluation of the in situ glycan editing strategy was based on growth factor receptors, we next sought to assess the use of this technique to modulate the function of ion channels. Human *ether-à-go-go*-related gene (hERG) channel is a potassium channel that belongs to voltage gated ion channels,^[24] which plays important roles in electrical signaling by repolarizing the cell membrane in the heart.^[25] hERG has only one extracellular N-linked glycosylation site^[26] and its gating properties is known to be related to protein glycosylation.^[27]

To determine if the in situ added monosaccharides could modulate hERG channel gating properties, HEK-293 cells stably expressing hERG channels were subjected to in situ Sia or Fuc editing followed by voltage clamp experiment to record whole-cell current. A standard depolarizing pulse protocol for the hERG channel was employed (Figure 4a). The current traces of control untreated cells and in situ glycan edited cells were recorded (Supporting Information, Figure S9a, S10a). From the *I-V* curve measured at the end of the depolarizing clamp steps, no significant changes of current were observed in cells treated with in situ Sia or Fuc editing (Supporting Information, Figures S9b, S10b). Then we compared the steady-state activation (SSA) curve of hERG channels between the control and the in situ glycan editing group to study the voltage dependence of activation. SSA curves based on the tail currents were measured at the initial point of the repolarizing clamp steps. As shown in Figure 4b, there was a significant depolarizing shift in sialylation group, with the mean $V_{1/2}$ shifting from -4.3 mV to 1.6 mV ($n = 6-8$, $p < 0.01$). By contrast, no significant difference of SSA relationships between the control group and fucosylated group was observed (Supporting Information, Figure S10c). These data suggested that in situ Sia editing

modulates the hERG channel gating by depolarizing its activation voltage—more depolarized potential is required to activate hERG channel after in situ Sia editing.

Although in situ Fuc editing had no apparent impact on the voltage dependent-activation of hERG channel, it was detected to have a major influence on the hERG maximum current by whole-cell voltage-clamp experiments. The patch was initially maintained in the whole-cell mode and then excised into the normal buffer. GDP-Fuc and α 1,3 FucT were freshly added in the buffer and continuously perfused to the cell for 40 min. During this procedure, maximal tail currents of hERG channels evoked with $V_h = 40$ mV were recorded every 15 s. As soon as in situ Fuc editing was applied, normalized hERG maximal current was suppressed (Figure 4c). Compared with the control group, the fucosylated group exhibited accelerated decreasing rate of normalized hERG maximal tail current during the 40-min perfusion. As a result, the normalized hERG maximal tail current of fucosylated group is approximately 25% lower than that of control group at the end of the 40-min perfusion (Figure 4c, bottom), indicating that in situ Fuc editing suppressed hERG channel activity by inhibiting its current.

In 1979, Paulson and co-workers demonstrated that sialyltransferases with distinct specificities could be used to restore Sia α 2-6Gal, Sia α 2-6GalNAc, or Sia α 2-3Gal epitopes onto the surface of previously de-sialylated erythrocytes.^[28] However, this method has not received wide attention because of the limited availability of recombinant glycosyltransferases that can catalyze glycan transfer on the cell surface. One exception to this observation is the human α 1,3-fucosyltransferase VI. Demonstrated by McEver, Sackstein, and others, this enzyme actively transfers fucose from GDP-Fuc to create sialyl Lewis X epitopes on the cell-surface to direct cord blood cell engraftment and homing of mesenchymal stem cells to bone.^[29] By contrast, using glycosidases for global glycan editing is a routine practice owing to the readily availability of these enzymes from commercial sources.^[30]

In the current study, we demonstrated that in situ glycan editing is a powerful approach for transiently modulating cell-surface receptor signaling and ion channel properties. Via this approach, sialic acid, fucose, and their analogues can be added in situ onto the cell surface in a linkage specific manner. Sialic acid and fucose added in situ exhibited similar effects in suppressing EGFR and FGFR dimerization and downstream signaling. Nevertheless, they were found to have distinct impact on hERG channel gating and activity. In situ Sia editing modulates hERG channel activation by depolarizing SSA relationships whereas in situ Fuc editing inhibits the maximal hERG tail current. It is possible that the different modulating patterns observed for in situ Fuc and Sia editing are caused by different locations and charges of the newly added α 1,3-fucosides and α 2,3-sialosides. It is worth noting that the fraction of receptors modified by in situ glycosylation has not been determined in this work. Currently in our laboratory we are developing a method for the quantitative measurement of the modified receptors and evaluating the feasibility of applying this method to modulate receptor signaling pathways in more complex systems such as cultured organoids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

In situ glycan editing of cell-surface glycans. Specific labeling of glycans terminated with galactose is achieved by in situ Sia editing with CMP-SiaNAI followed by reaction with an azide probe via CuAAC. Modulation of cell signaling is achieved by remodeling cell-surface glycans via in situ Sia editing with CMP-Sia or in situ Fuc editing with GDP-Fuc.

Figure 2.

Determine the labeling specificity of recombinant ST3Gal-IV in CHO cell mutants via western blot, flow cytometry, and imaging analysis. a) CHO cell mutants were treated with CMP-SiaNAI or CMP-Sia (500 μ M) and ST3Gal-IV (50 μ g mL⁻¹) for 30 min. The labeled cells were then reacted with biotin-Az via CuAAC, lysed, and probed with anti-biotin antibody (top). The Coomassie blue stain proved equal loading amount (bottom). b) CHO cell mutants were biotinylated as described above. The labeled cells were probed with streptavidin-Alexa Fluor 488 for flow cytometry analysis ($n = 3$). c) Imaging of cell-surface glycans terminated with galactose. Lec2 cells stained with chloro-methyl fluorescein diacetate (CMFDA, red) and unstained Lec8 cells were mixed at a 1:4 ratio and cultured for 3 days. The cells were treated with ST3Gal-IV and CMP-Sia or CMP-SiaNAI, then stained with Alexa Fluor 647-Az (green) and Hoechst 33342 (blue). Scale bars: 20 μ m.

Figure 3.

Cell-surface in situ glycan editing suppresses EGFR signaling. a) A549 cells were labeled with CMP-SiaNAI or CMP-Sia and ST3Gal-IV, then reacted with biotin-Az via CuAAC. EGFR pull-down from cell lysate were resolved and probed with anti-biotin antibody (left); biotinylated proteins pull-down from cell lysate were resolved and probed with anti-EGFR antibody (right). b) Starved A549 cells were treated with or without in situ Sia or Fuc editing and stimulated with EGF. Lysates were prepared and analyzed by Western blotting ($n=3$, $*p<0.05$). c) Wound-healing assay for the effects of EGF, in situ Sia editing and sialylation inhibitor on cells migration. A549 cells were treated and wounded, the summarized migration area were measured using ImageJ ($n=6$, $**p<0.01$).

Figure 4.

Cell-surface in situ glycan editing modulate the hERG channel functions. a) The pulse protocol for whole-cell voltage clamp experiment. Cells were held at -80 mV and depolarized to pre-pulse potential ranging from -60 to $+40$ mV with 10 -mV increments for 4 s, followed by a -40 mV repolarizing step. Arrows indicate points at which currents were measured (solid: for $I-V$ curves, dash: for SSA curves). b) SSA curves for hERG channels in untreated and in situ Sia edited cells ($n = 6-8$). Lines are fits of the data to single Boltzmann distributions. c) The normalized maximal tail current for hERG channels in untreated and in situ Fuc edited cells ($n = 6$, $**p < 0.01$).